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1. **Your reference** — 59.82765

2. **Patent application number** *(The Patent Office will fill in this part)* — 0329449.3

3. **Full name, address and postcode of the or of each applicant** *(underline all surnames)*

OmniCyte Ltd
21 Wilson Street
London
EC2M 2TD

Patents ADP number *(if you know it)* — 8776023001

If the applicant is a corporate body, give country/state of its incorporation — United Kingdom

4. **Title of the invention** — Stem Cells

5. **Name of your agent** *(if you have one)* — Frank B. Dehn & Co.

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

179 Queen Victoria Street
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11. I/We request the grant of a patent on the basis of this application.

Signature Frank B Dehn & Co. Date 19 December 2003

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Rebecca Gardner
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Stem Cells

The present invention relates to stem cells, in particular to a new type of adult stem cells isolatable from bone marrow and blood.

Stem cells can produce new cells to repair damage to any tissue in the body and therefore have immense potential for all types of regenerative medicine. Stem cells are present in all body tissues and organs but some, like bone marrow and blood, are more accessible than others, like liver and brain. However, stem cells exist in very small numbers in marrow and blood, and need to be extracted then increased in number ("expanded") before they can be used clinically. Currently, many attempts are being made to accomplish the aim of providing stem cells in sufficient numbers to perform tissue-specific stem cell transplantation.

Efforts have focussed on the bone marrow as a source of stem cells. Evidence to date suggest that the bone marrow contains two types of stem cells - haemopoietic stem cells (HSC) responsible for producing blood, and mesenchymal stem cells (MSC) capable of producing cells belonging to a limited range of body tissues. MSC can not be precisely defined or isolated and show a limited capacity to provide multiple cell types. They form osteoblasts, chondroblasts and adipocytes in response to stimulation in culture but there are many cell types, such as hepatocytes, which they cannot form. Prolonged culture of MSC results in the outgrowth of a subpopulation of cells referred to as multipotent adult progenitor cells (MAPC) which to date appear to have the broadest potential for tissue regeneration. However, the fact the prolonged tissue culture and many cell divisions are required before MAPC emerge, means first that they

may have accumulated genetic damage and second, that it is impossible to be certain that they represent normal cellular components of the bone marrow. They may, in fact, be a tissue culture artefact. These important considerations potentially contra-indicate the clinical use of MAPC. MAPC can form cells with endodermal, ectodermal or mesodermal markers but, significantly, do not produce haemopoietic cells in culture.

Using differentiated cells derived from stem cells to produce their natural protein products has advantages over the use of cells to produce recombinant proteins, particularly because of the capability for appropriate glycosylation and post-translational modification of the protein product.

The present inventors have now identified a new type of pluripotent stem cell which can be directly isolated from bone marrow and blood e.g. peripheral or taken from the umbilical cord and have the unique ability to differentiate into ectodermal, mesodermal and endodermal cells. These cells are thus clearly multipotent if not totipotent. Thus the adult, i.e. non-foetal, stem cells described herein provide a novel source of cells for tissue transplantation that may be used in an autologous (self-to-self) manner. Further, as is described below, these stem cells do not require prolonged tissue culture.

Thus, in one aspect the present invention provides an isolated adult stem cell population wherein said stem cells are $CD34^+$, capable of self regeneration and capable of differentiation into ectodermal, mesodermal and endodermal cells.

These stem cells are further characterised by their ability to adhere to plastic (e.g. the plastic of standard tissue culture vessels) during culturing. Suitable vessels are those manufactured by Corning Incorporated, New York, USA.

The stem cells of the invention may be further characterised by the fact that they do not require feeder layers, i.e. cells (typically inactivated by gamma irradiation which supply important metabolites without further growth or division of their own) which support the growth of the stem cells. Thus, preferably, a feeder layer is not used during culturing of the stem cells.

One primary characterising and particularly advantageous feature of these stem cells is their ability to differentiate into a very wide variety of different cell types including ectodermal, mesodermal and endodermal cells. Thus, these stem cells of the invention can differentiate into cell types which are developmentally derived from the three germ layers of the embryo; ectoderm, mesoderm and endoderm; for example haemopoietic and muscle cells which are derived from the mesoderm; nerve or epithial cells from the ectoderm; and glandular epithelium or hepatocytes from the endoderm.

The cell population is 'isolated' in that it is substantially free of other cell types, in particular of cells which express CD33, CD38, HLA/DR, CD19 and CD3. Also, the population is substantially free of cells dedicated to a particular lineage and/or cells carrying markers associated therewith. Preferably the population has less than 20%, more preferably less than 10%, e.g. less than 5% of lineage committed cells. It may assist in the isolation of the present stem cell population to combine both negative selection (removal of cells) and positive selection (isolation of cells), in both cases antibody binding may be used.

Stem cells are thought to be manufactured in the adult bone marrow but are also found in the blood. The present stem cells may be collected from either of these sources according to standard sampling techniques. Blood samples are preferably obtained following stem cell mobilisation with G-CSF to increase the numbers of stem

cells in the circulation. For example, 5 μg/kg body weight/day may be administered subcutaneously for 5 days. It is also possible to obtain direct bone marrow samples, e.g. through aspiration.

Bone marrow cells may be obtained from a source of bone marrow, e.g., iliac crests, tibiae, femora, spine, or other bone cavities. Conveniently bone marrow may be aspirated from the bone in accordance with conventional techniques. Other sources of the stem cells include blood, including adult peripheral blood and umbilical cord blood.

Various techniques may be employed to separate the cells by initially removing cells of dedicated lineage. Monoclonal antibodies are particularly useful for identifying markers (surface membrane proteins) associated with particular cell lineages and/or stages of differentiation. The antibodies may be attached to a solid support to allow for crude separation. The separation techniques employed should maximize the retention of viability of the fraction to be collected. For "relatively crude" separations, that is, separations where up to 10%, usually not more than about 5%, preferably not more than about 1%, of the total cells present having the marker, may remain with the cell population to be retained, various techniques of different efficacy may be employed. The particular technique employed will depend upon efficiency of separation, cytotoxicity of the methodology, ease and speed of performance, and necessity for sophisticated equipment and/or technical skill.

Procedures for separation may include magnetic separation, using antibody-coated magnetic beads, affinity chromatography, cytotoxic agents joined to a monoclonal antibody or used in conjunction with a monoclonal antibody, e.g., complement and cytotoxins, and "panning" with antibody attached to a solid matrix, e.g.,

plate, or other convenient technique. Techniques providing accurate separation include fluorescence activated cell sorters, which can have varying degrees of sophistication, e.g., a plurality of color channels, low angle and obtuse light scattering detecting channels, impedance channels, etc.

Conveniently, the antibodies may be conjugated with markers, such as magnetic beads, which allow for direct separation, biotin, which can be removed with avidin or streptavidin bound to a support, fluorochromes, which can be used with a fluorescence activated cell sorter, or the like, to allow for ease of separation of the particular cell type. Any technique may be employed which is not unduly detrimental to the viability of the remaining cells.

Preferably the mononuclear fraction of the blood or bone marrow sample is separated using a Lymphoprep™ (Axis Shield) density gradient. $CD34^+$ cells can be separated from the mononuclear fraction using MiniMACS (Miltenyi Biotec) technology.

The stem cells of the present invention can be further characterised by the methods used to obtain them, thus the cells are obtainable by a combined affinity purification and selection by adherence method. More particularly, the cells can be labelled with CD34 monoclonal antibody (MAb) and then with (para)magnetic beads which themselves bind to the CD34 MAb. Alternatively, beads which are themselves labelled with the CD34 MAb may be used which bind to the cells. The labelled or bound cells can then be applied to a column and held in place by a magnet; unlabelled cells will be eluted and labelled cells released by removing the magnet (or by removing the column from the magnet).

The thus released $CD34^+$ cells can then be incubated in tissue culture plastic vessels for at least 2 hours, preferably at least 3 hours e.g. 3-5 hours, with

non-adherent cells removed by washing with HBSS (Hanks balanced salt solution). The adherent $CD34^+$ cell population are the stem cells of the present invention and comprise less than 1% of the total $CD34^+$ population. They are preferably a substantially homogenous population, generally uncontaminated by other stem cell subpopulations. Typically less than 5%, preferably less than 3% of the cells collected are other than the stem cells of the present invention. 'Adherent' cells are defined as those which are able to resist vigorous washing three times without detaching. The advantageous properties of the adherent subset of $CD34^+$ cells are surprising as the adherent cells would usually be discarded when culturing cells.

The stem cells of the invention are capable of self-regeneration, i.e., in accordance with standard definitions of stem cells, each stem cell is capable of division to form further stem cells, as well as differentiation to a wide variety of different cell types.

The stem cells of the invention are further characterised as $CD34^+$, i.e. expressing the antigen CD34, a glycoprotein marker found, but not exclusively so, on stem cells, in particular the stem cells manufactured in the bone marrow (HSC and MSC). The cells are also $Thy\text{-}1^+$.

More particularly, the cells may be characterised as $CD34^+$, $Thy\text{-}1^+$, $CD38^-$, $CD33^-$ and $HLA\text{-}DR^-$.

The stem cells are lymphocyte-like in that they are round mononuclear cells and rather small with a high nucleus: cytoplasm ratio. Such a morphology is associated with primitive stem cells.

They are characterised by an ability to produce differentiated cells in less than 16, e.g. 12-14 days in culture. Preferably differentiation is observed in less than 14 days, e.g. less than 10 days, more preferably in less than 7 days, even 4-5 days.

The stem cells of the invention can be further characterised as obtainable by:

(i) subjecting haemopoietic tissue (i.e. blood or a bone marrow sample) to density gradient separation;
(ii) exposing low density cells to an affinity ligand for CD34 (preferably attached to paramagnetic beads);
(iii) recovering cells attached to said CD34 ligand;
(iv) exposing the $CD34^+$ subpopulation to tissue culture grade plastic; and
(v) recovering $CD34^+$ cells adherent to the plastic.

The stem cells of the invention may be from any animal, e.g. laboratory, livestock or companion animal; preferably primate and most preferably from humans.

In a further embodiment the present invention provides a culture comprising:

(i) an isolated adult stem cell population wherein said stem cells are $CD34^+$, capable of self regeneration and capable of differentiation into ectodermal, mesodermal and endodermal cells; and
(ii) a medium capable of supporting the growth of said stem cells.

Once stem cells have been isolated, they may be propagated by growing in conditioned medium from stromal cells, such as stromal cells that can be obtained from bone marrow, fetal thymus or fetal liver, and are shown to provide for the secretion of growth factors associated with stem cell maintenance, coculturing with such stromal cells, or in medium comprising maintenance factors supporting the proliferation of stem cells, where the stromal cells may be autologous, allogeneic or xenogeneic. Before using in the coculture, the mixed

stromal cell preparations may be freed of haemopoietic cells employing appropriate monoclonal antibodies for removal of the undesired cells, e.g., with antibody-toxin conjugates, antibody and complement, etc. Alternatively, cloned stromal cell lines may be used where the stromal lines may be allogeneic or xenogeneic. Thus, reference above to "medium" includes cells such as stromal cells.

In a further aspect, the invention provides a method of isolating an adult stem cell population wherein said stem cells are $CD34^+$, capable of self regeneration and capable of differentiation into ectodermal, mesodermal and endodermal cells, which method comprises taking a sample of blood or bone marrow from a subject and extracting said cell population therefrom. Preferred extraction steps are discussed above and in the case of blood sampling there will typically be a first step of stem cell mobilisation which is preferably performed by administering G-CSF to the subject.

The stem cells of the present invention have utility in research contexts, for example in detecting and evaluating growth factors relevant to stem-cell regeneration. The stem cells may also be of direct utility in the treatment of genetic diseases through gene modification or replacement in autologous stem cells. In particular the cells may be used in the treatment of diseases associated with haemopoietic cells, such as β-thalassemia and sickle cell anemia, where a wild-type gene is introduced into the stem cells. Thus in a further aspect the invention provides an isolated adult stem cell population wherein said stem cells are CD34+, capable of self regeneration and capable of differentiation into both haemopoietic and mesenchymal cells which further incorporate a therapeutic gene, for use in therapy. Equally, the invention provides a method of gene therapy comprising administering to a patient in need thereof a population of stem cells wherein said stem

cells are $CD34^+$, capable of self-regeneration and capable of differentiation into ectodermal, mesodermal and endodermal cells and incorporate a therapeutic gene. Suitable therapeutic genes will include a wild-type version of a gene which is defective in the patient or a drug resistance gene.

Without additional therapeutic genes the stem cells still have therapeutic utility, e.g. in regenerating the haematopoietic system of a patient deficient in stem cells.

Further utilities of great interest relate to the generation of different differentiated cell types from the stem cells of the invention. As shown in the Figures hereto, it has been possible to generate in advantageously short timescales mesenchymal, haemopoietic, endothelial, epithelial, tube-forming and dendrite-forming cells. The preparation of haemopoietic and mesenchymal cells being particularly preferred. Cells have been observed after 4-5 days with the appearance of liver, nerve, mesenchymal, endothelial, epithelial and and haemopoietic cells.

The stem cells are cultured with a cocktail of different cytokines, depending on the desired cell type. The cocktail will typically comprise G-CSF, GM-CSF, IL-3 and stem cell factor, with HGF and FGF being added to stimulate differentiation of hepatocytes; nicotinamide and LY294002 to stimulate differentiation to pancreatic cells and FGF and dibutyryl cyclic AMP to encourage production of nerve cells. Other growth factors are known to the skilled man to be important in the differentiation of other cell types such as bone, cartilage, skeletal and cardiac muscle, kidney, lung, nerve, skin and endocrine tissue.

Thus, in a further aspect, the invention provides a method of producing a target cell type which comprises culturing the stem cells of the invention with a

plurality of growth factors. As described in the Examples and shown in the Figures, successful differentiation may be shown by visual inspection, flow cytometry or immunophenotyping. Cells expressing albumin, α-fetoprotein, α-antitrypsin and hepatocyte growth factor receptor (HGF receptor) (properties of hepatocytes), vimentin (skeletal muscle and neuronal cells) and smooth muscle actin (muscle cells) have, for example, been confirmed.

The differentiated cells can then be transplanted into a patient in need thereof. Of particular benefit is the potential to generate differentiated cells for cell or tissue transplantation that are derived from the patient's own stem cells. Such techniques are known in the art and use different routes of administration according to the particular target tissue. The liver for example is able to regenerate itself following introduction of a population of healthy liver cells, where the liver has been damaged, e.g. as a result of Hepatitis infection or alcohol abuse. Immune suppression may be treated by administration of lymphocytes, muscle wasting by the introduction of skeletal muscle cells, diabetes through transplanting pancreatic cells and so on.

The cells may be administered in a localised manner, e.g. injected directly into a target organ such as the liver. Alternatively, the cells may be administered at a site remote from the target site, e.g. by intravenous delivery. Tissue targeting may be achieved by forming a complex between the generated cell types and a targeting ligand, such as monoclonal antibodies, cell adhesion molecules and their ligands, cytokine, chemokine and toll-like receptors and their ligands. Such 'complexes' include cells which express the targeting ligand on their cell surface.

In one preferred embodiment the transplanted cells are adapted to be tracked *in vivo*, i.e. they incorporate a labelling moiety which means the location of the cells in the body can be identified. Conveniently the cells will incorporate iron compounds e.g. iron oxide, and then MRI can be used to confirm the location of the transplanted cells, in particular to confirm whether they have reached their target tissue. As described in Example 4, the MR agent Resovist® is a suitable iron containing compound which can be taken up by the cells on incubation therewith.

Thus, according to further aspects, the invention provides differentiated cell populations prepared according to the method defined above and such cells for use in therapy, as well as methods of medical treatment which comprise administration of these differentiated cell populations to a patient. In particular, the invention comprises a method for the transplantation of a population of differentiated cells, the method comprising:

(i) culturing a population of the stem cells of the present invention with a plurality of growth factors so as to cause differentiation thereof; and

(ii) transplanting said differentiated cells into a patient.

Preferably the patient is human and also preferably, the stem cells which are cultured to produce the differentiated cells are from the patient.

The short time required from taking a sample from a patient and growing up differentiated cells for administration is a particular benefit provided by the present invention.

The stem cells of the invention and differentiated cells derived therefrom may also be used in the *in vitro* production of proteins of interest. Thus in a further

aspect the invention provides an *in vitro* method of protein production which comprises culturing the stem cells of the invention or a differentiated cell line derived therefrom and then harvesting the cells and recovering one or more of the proteins expressed by said cells.

Animal cells have become the predominant protein expression system for *in vitro* production of target proteins, particularly therapeutic agents, because of their ability to perform post-translational modification (e.g. glycosylation) of proteins. The stem cells of the invention and their differentiated progeny can be used in the production of most if not all proteins of therapeutic interest, such as EPO, growth factors, protein hormones such as insulin etc. or synthetic proteins. The cells may be genetically modified in order to provide or enhance production of a particular target protein. However it is a particularly desirable feature of the cell types enabled by the present invention that differentiation to an appropriate cell type (e.g. parenchymal cells) can be performed such that the cells naturally produce the target protein without the need for genetic engineering.

The above described uses of the cells of the invention (stem and differentiated) are also applicable to non-protein products such as steroids, in both cases, suitable culturing and harvesting techniques are known to the skilled man.

The stem cells of the invention have the necessary cellular machinery to propagate vectors such as adenovirus, retrovirus, adeno-associated virus etc.; this is an essential step for current good manufacturing practice (cGMP) preparation of such vectors. Thus, in a further aspect, the present invention provides the use of the stem cells of the invention as defined and described herein in vector, particularly viral vector, production.

Alternatively viewed, the invention provides a method of vector production wherein the vector of interest is propagated in a stem cell of the invention as defined and described herein. The vector (a transmissable agent) is typically a viral vector such as an adenovirus, retrovirus or adeno-associated virus.

The invention will be further described in the following non-limiting Examples and with reference to the Figures in which:

Figures 1-10 are photographs showing the stem cells of the invention and their differentiation over time into mesenchymal cells (Fig. 3, 5 and 6), haemopoietic cells (Fig. 4), epitheliod cells (Figs. 7 and 8), tube-forming cells (Fig. 9) and dendrite-forming cells (Fig. 10).

Figure 11 is a photograph showing how $CD34^+$ cells were able to take up Resovist® (Schering AG) according to the protocol described in Example 4. According to this figure the individual spots represent the following:

1. 10^6 cells, 0.25 mmol Resovist, overnight incubation
2. 10^6 cells, 0.25 mmol Resovist + beads, overnight incubation
3. 10^6 cells, beads, overnight incubation
4. 10^6 cells, negative control (no staining)
5. $5x10^5$ cells, negative control (no staining)
6. $5x10^5$ cells, 0.25 mmol Resovist, overnight incubation
7. $5x10^5$ cells, 0.25 mmol Resovist + beads, overnight incubation
8. $5x10^5$ cells, beads, overnight incubation
9. 10^6 cells, 0.25 mmol Resovist, 2h incubation
10. 10^6 cells, 0.25 mmol Resovist + beads, 2h incubation
11. 10^6 cells, beads, 2h incubation
12. $5x10^5$ cells, 0.25 mmol Resovist, 2h
13. incubation $5x10^5$ cells, 0.25 mmol Resovist + beads, 2h incubation
14. $5x10^5$ cells, beads, 2h incubation

15. beads only, no cells.

Figure 12 are photographs showing the differentiation of stem cells into liver cells, as evidenced by the presence of the liver cell markers albumin and alpha fetoprotein.

Examples

Example 1. Cell extraction

Haematopietic cells were obtained from bone marrow or mobilised blood from normal individuals for transplantation purposes. The mononuclear fraction was separated from the whole using a Lymphoprep™ density gradient. CD 34^+ cells were separated from mononuclear fraction using MiniMACS technology. Cells were first labelled with CD34 monoclonal antibody and then with paramagnetic beads. Labelled cells were loaded onto a column held on a magnet, unlabelled cells were eluted and labelled cells released by removing the column from the magnet. The $CD34^+$ cells were incubated in tissue culture plastic vessels for at least 2h. Non adherent cells were removed by washing with HBSS.

Example 2. Cell culture

Cells (2×10^5/ml) were incubated in Methylcellulose medium containing serum, 100ng/ml granulocyte colony stimulating factor (G-CSF), 5ng/ml interleukin-3 (IL-3) 20ng/ml stem cell factor (SCF) and 1ng/ml granulocyte macrophage colony stimulation factor (GM-CSF). This resulted in heterogeneous populations of cells, which subsequently can be characterised. Selected individual populations were then targeted for differentiation using tailored cytokine cocktails.

Example 3. Flow cytometry and immunocytochemistry

Flow cytometry. The adherent population that had developed in the cultures was removed by scraping the dish. Cells were fixed in 4% paraformaldehyde and permeabilised. Cells were labelled with monoclonal

antibodies conjugated with FITC and analysed using Becton Dickinson flow cytometer.

Immunocytochemistry. The adherent population that had developed in the cultures was removed by scraping the dish. Cells were cytospun onto glass slides and fixed by methanol. Cells were labelled with monoclonal antibodies and visualised using the APAAP (alkaline phosphatase anti-alkaline phosphatase) reaction.

Results of flow cytometry and immunocytochemistry are shown in Table 1 below.

Table 1.

Antigen	Flow cytometry	Immunocytochemistry
Albumin	9.2%*	Large cells +ve**
Alpha feto protein	12.3%	Large cells +ve
Alpha 1 antitripsin	20.1%	N/A
cMET (HGF receptor)	34.9%	+ve
Smooth muscle actin	61.2%	Large cells +ve
GFAP(astrocytes)	57.4%	- ve
cKIT	22.1%	- ve
Vimentin	6.3%	Large cells +ve
hTERT	20.1%	- ve

* Denotes % of positive cells in the population
** Denotes that the large cells in the population were positive and the small cells were negative.

Albumin, alpha feto protein, alpha 1 antitripsin and cMET (HGF receptor) are liver cell markers; GFAP (astrocytes) is a brain cell marker; and smooth muscle actin is a marker for mesenchyme cells.

Figure 12 shows cells exhibiting liver cell markers after culturing for 12 days.

Example 4. Incorporation of iron oxide into cells and labelling cells with paramagnetic beads

$CD34^+$ (10^6 and $5x10^5$) cells were incubated with 0.25 mmol Resovist® (the brand name of Ferrixan, carboxy-dextran coated iron oxide nanoparticles available from Schering AG) for 2 and 24 hours at 37°C and analysed by MRI. In both cases, positive signal was obtained by MRI suggesting possible use of Resovist in detecting $CD34^+$ cells *in vivo*. The results of Figure 11 indicate that the particles can be taken up by the cells and therefore used in tracking the $CD34^+$ cells or their differentiated progeny as they move around the body or locate in target tissues.

The *in vitro* toxicity of Resovist was also tested by Trypan blue exclusion assay and proved to be non-significant (<4%).

The results of this experiment are shown in Fig. 11.

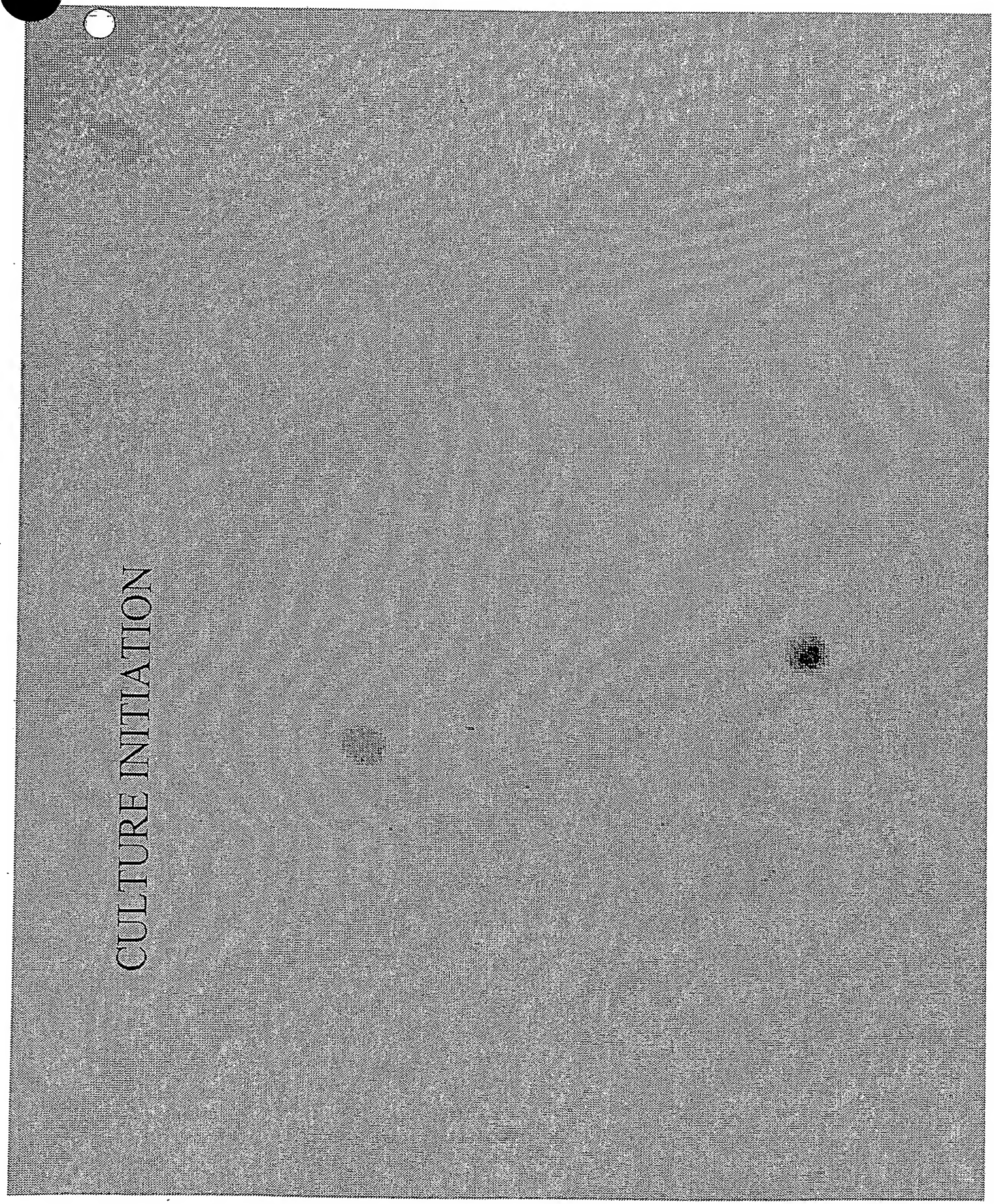

Fig. 1

Fig. 2

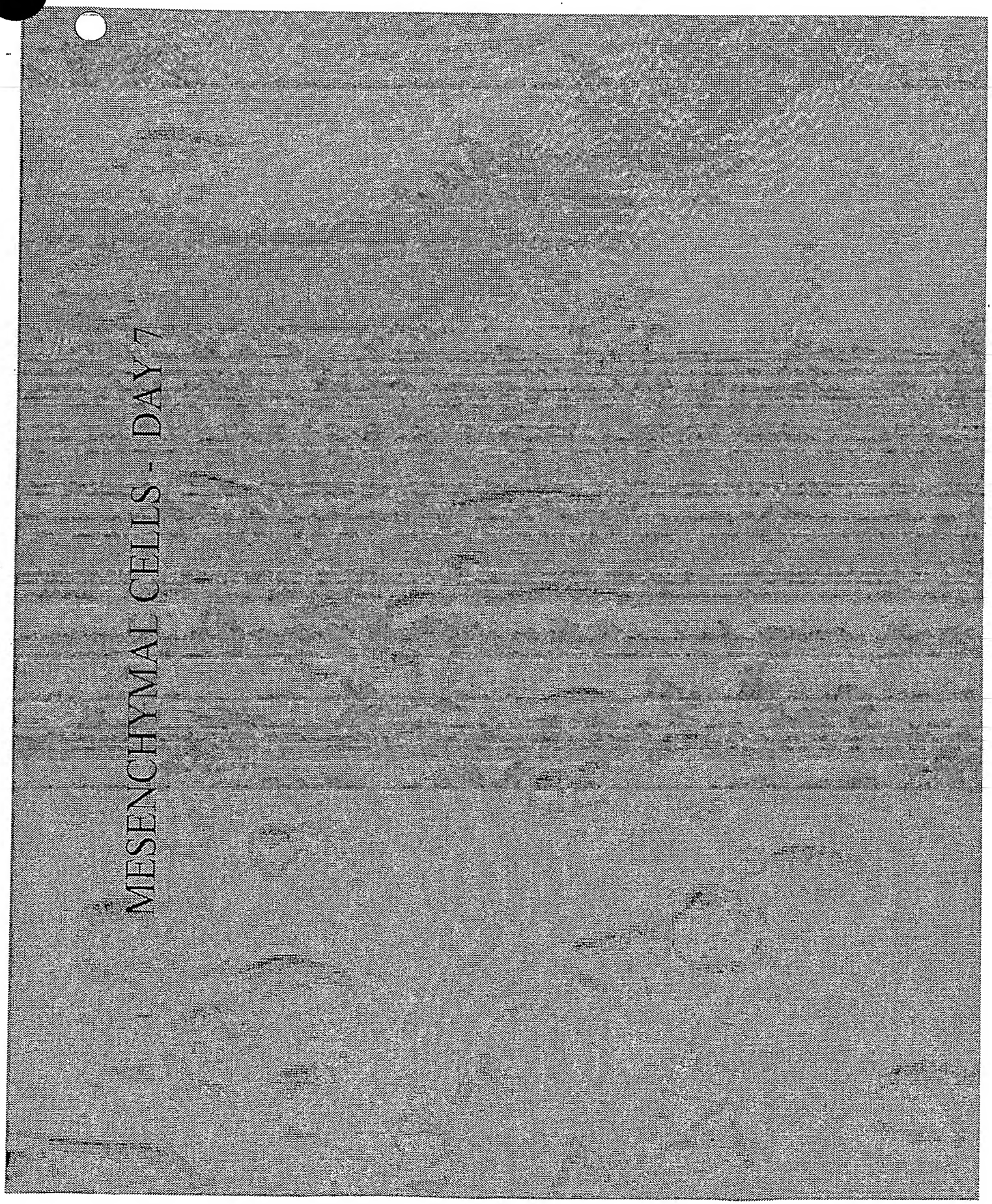

Fig. 3

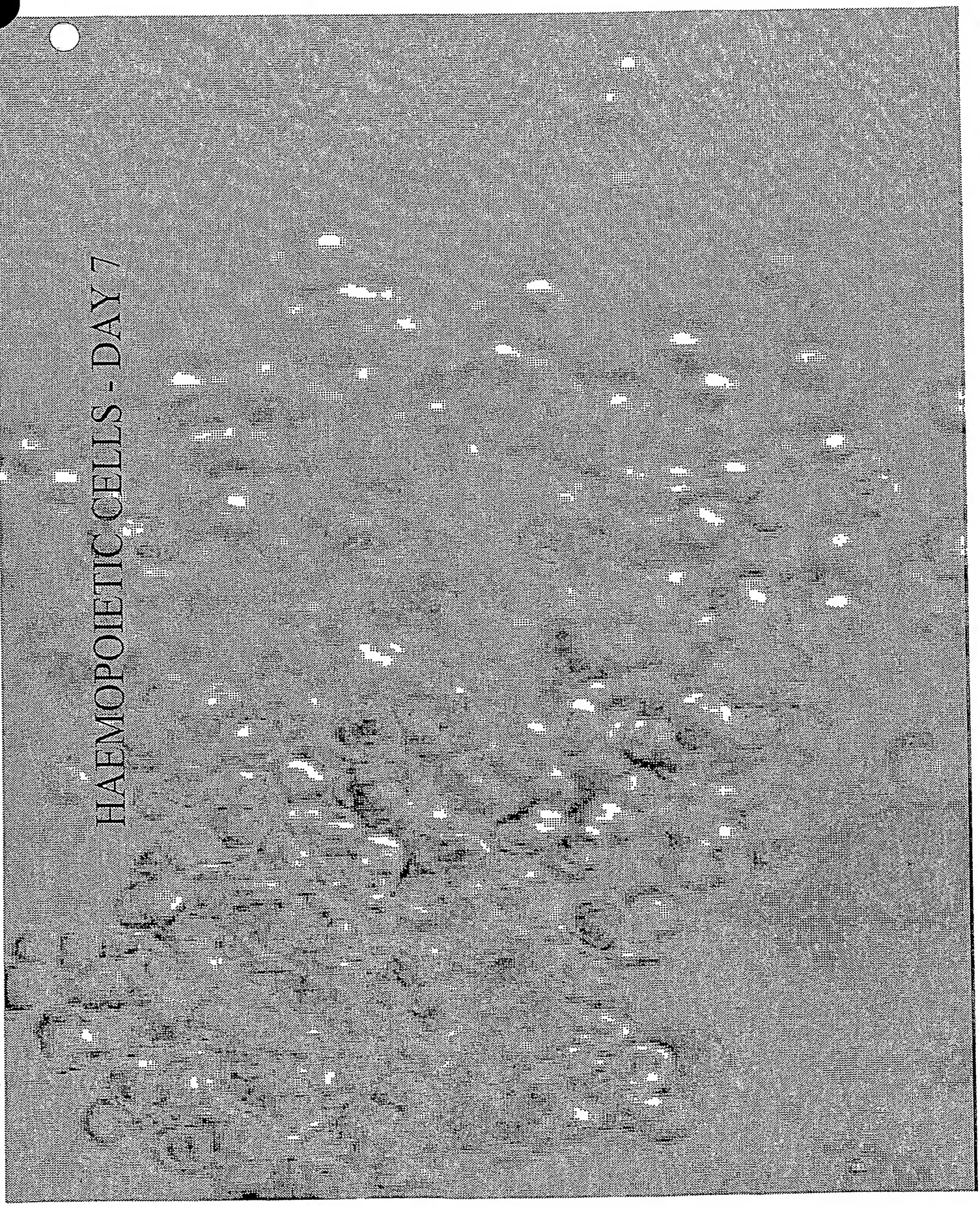

Fig. 4

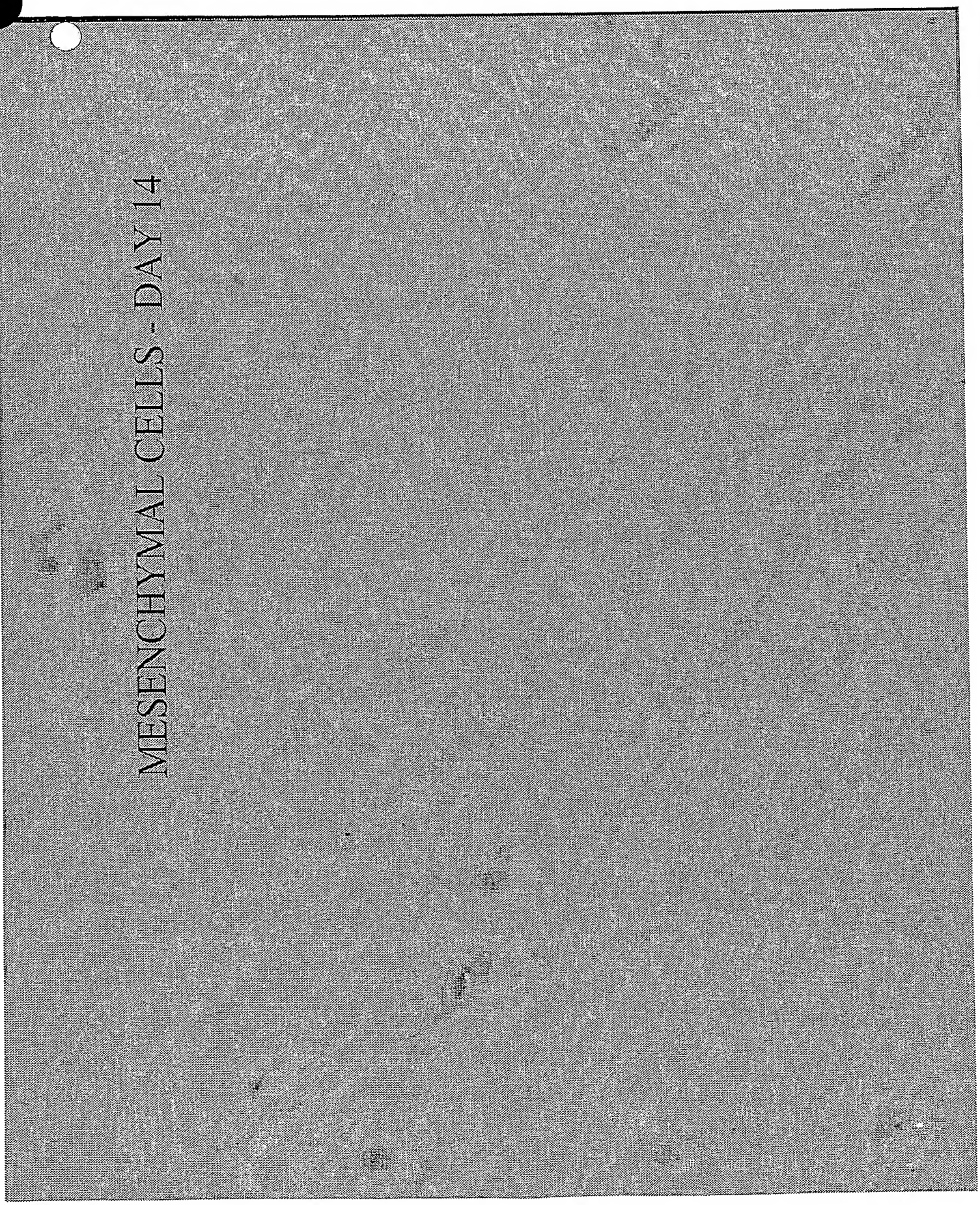

Fig. 5

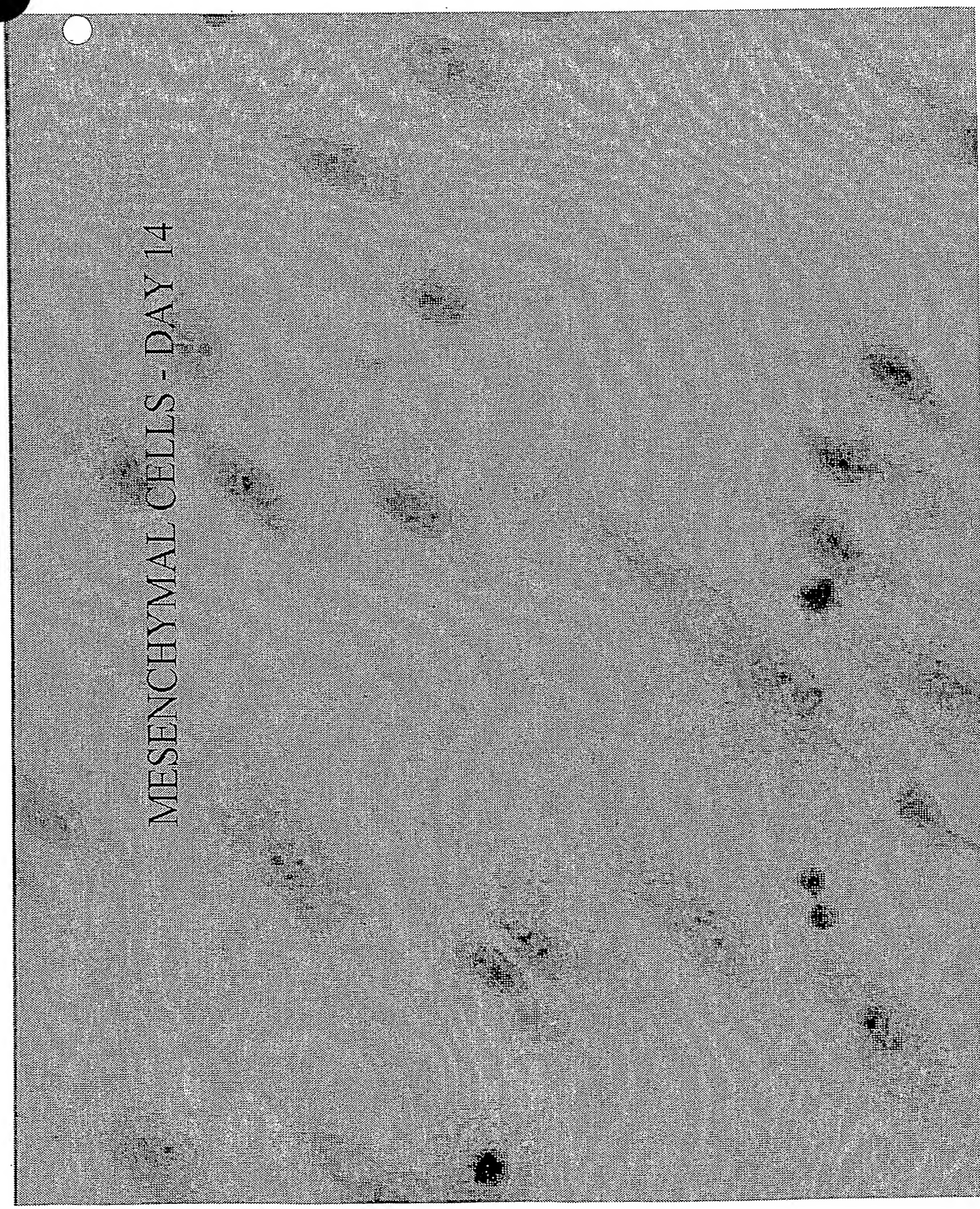

Fig. 6

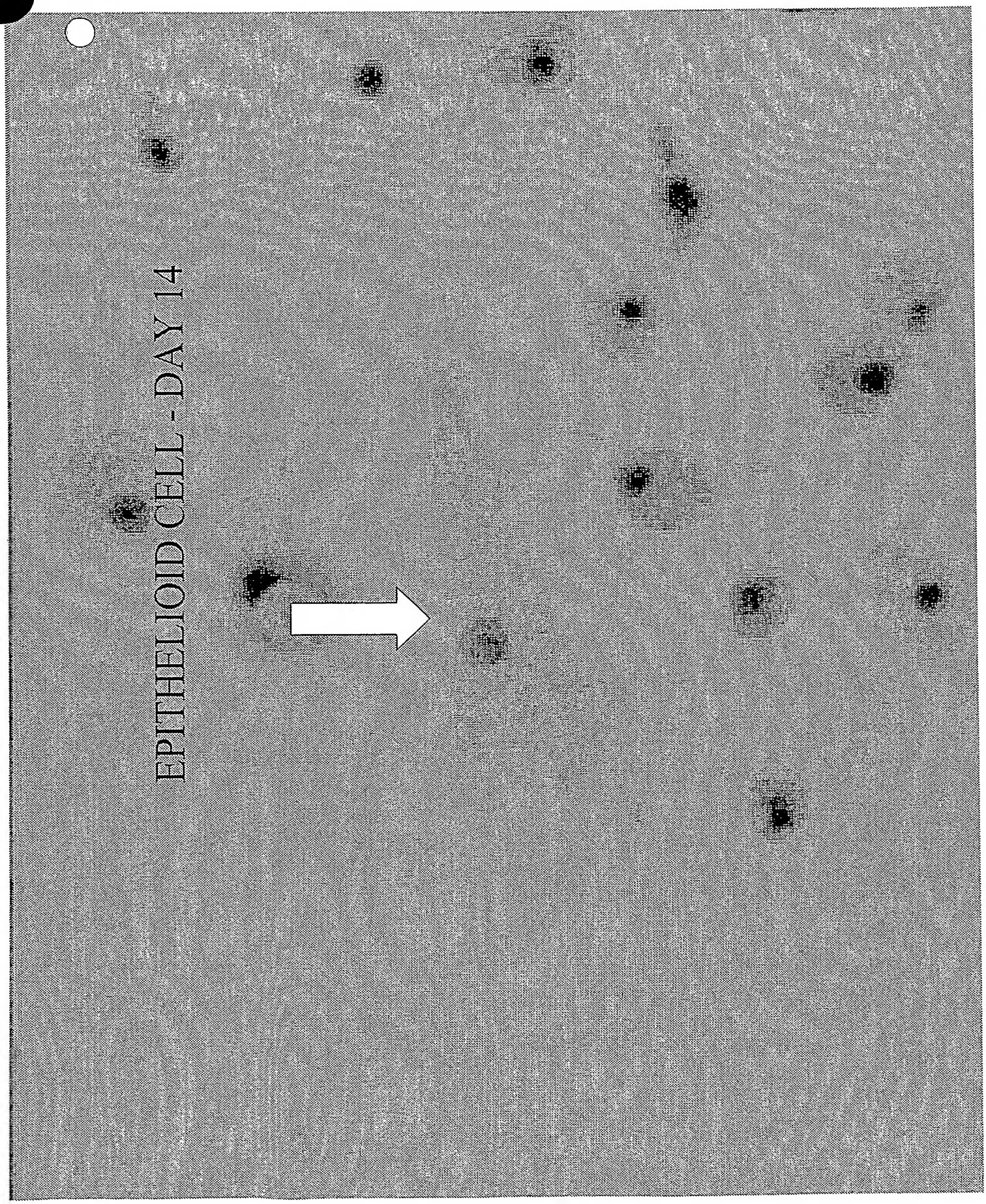

Fig. 7

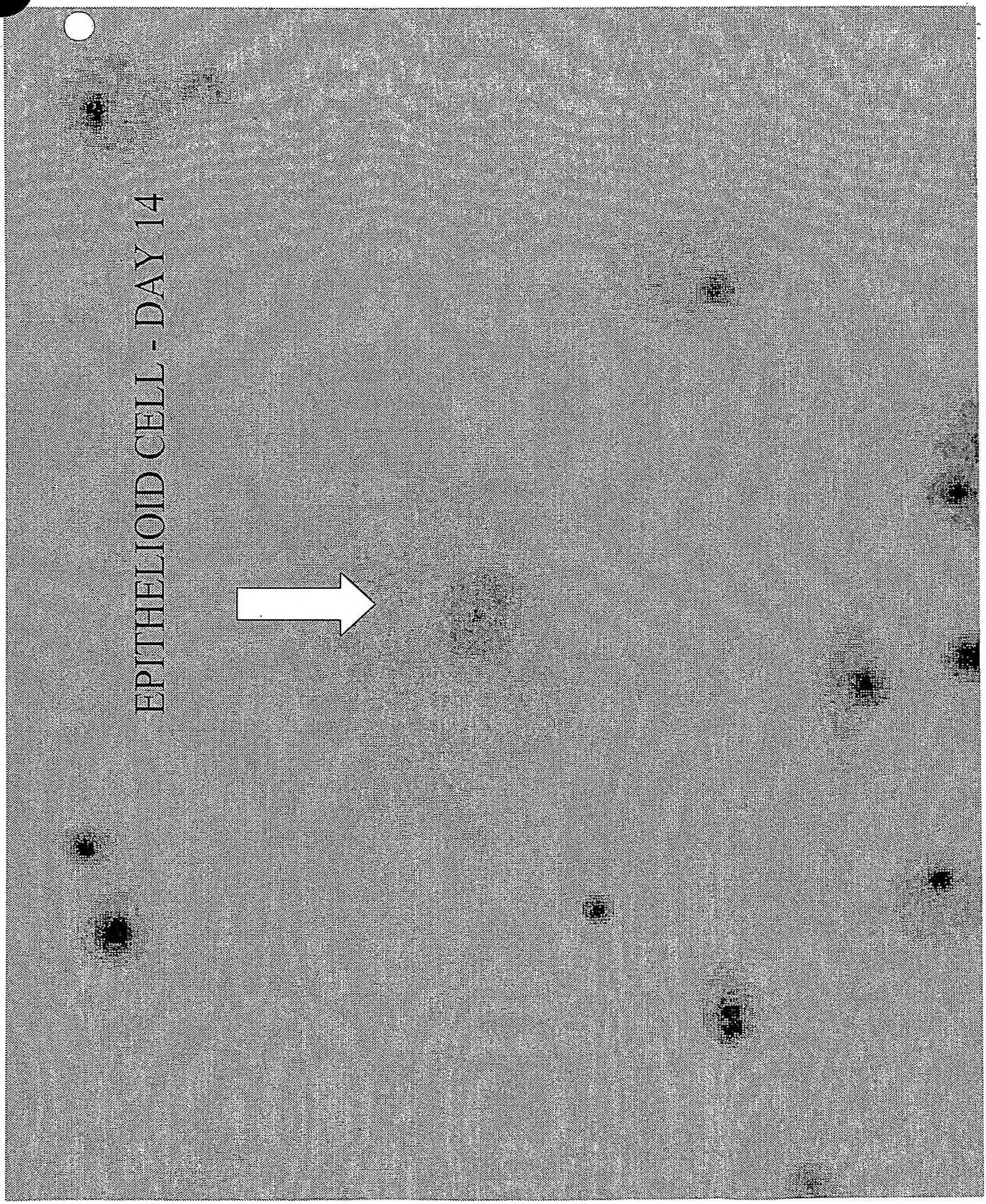

Fig. 8

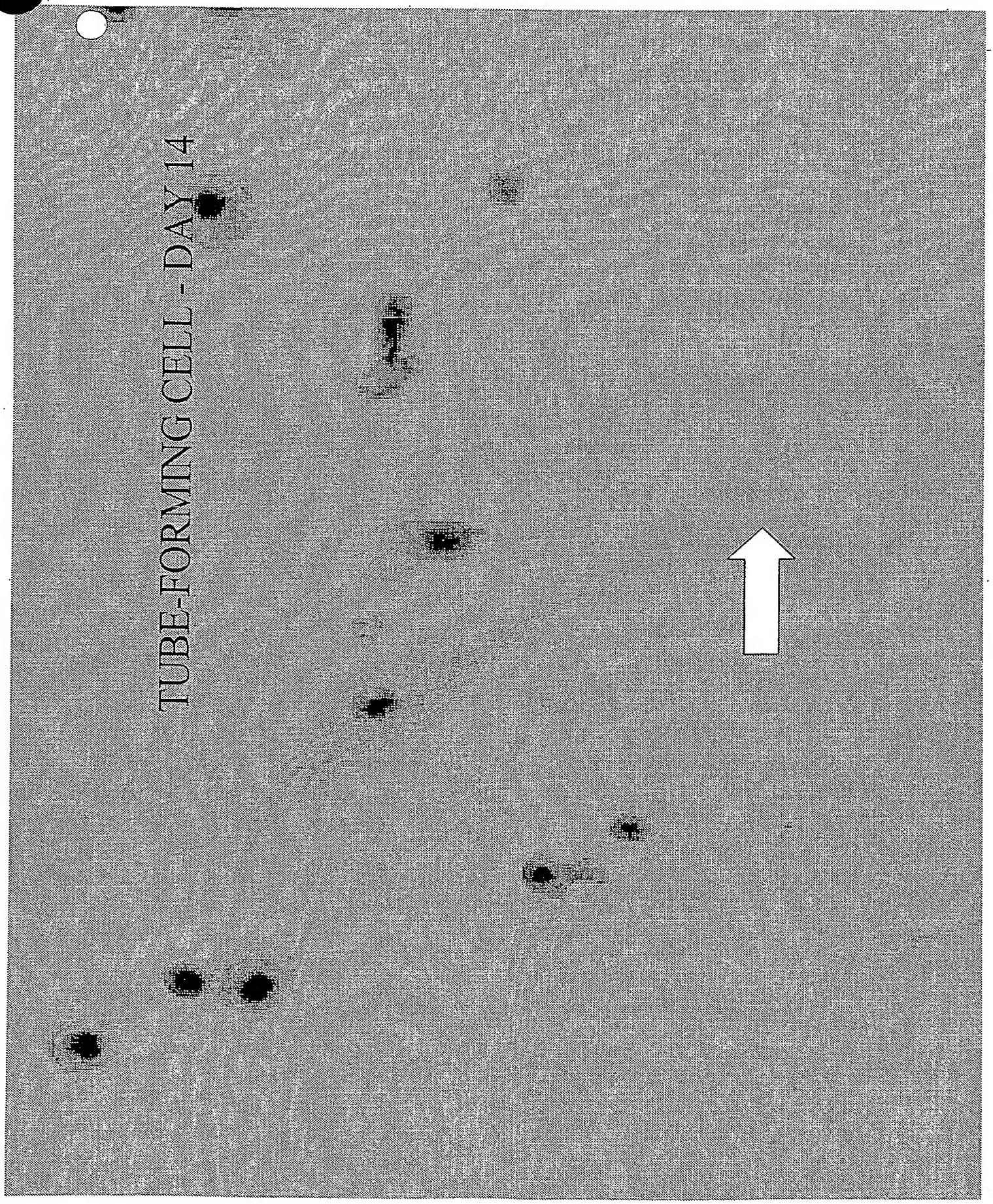

Fig. 9

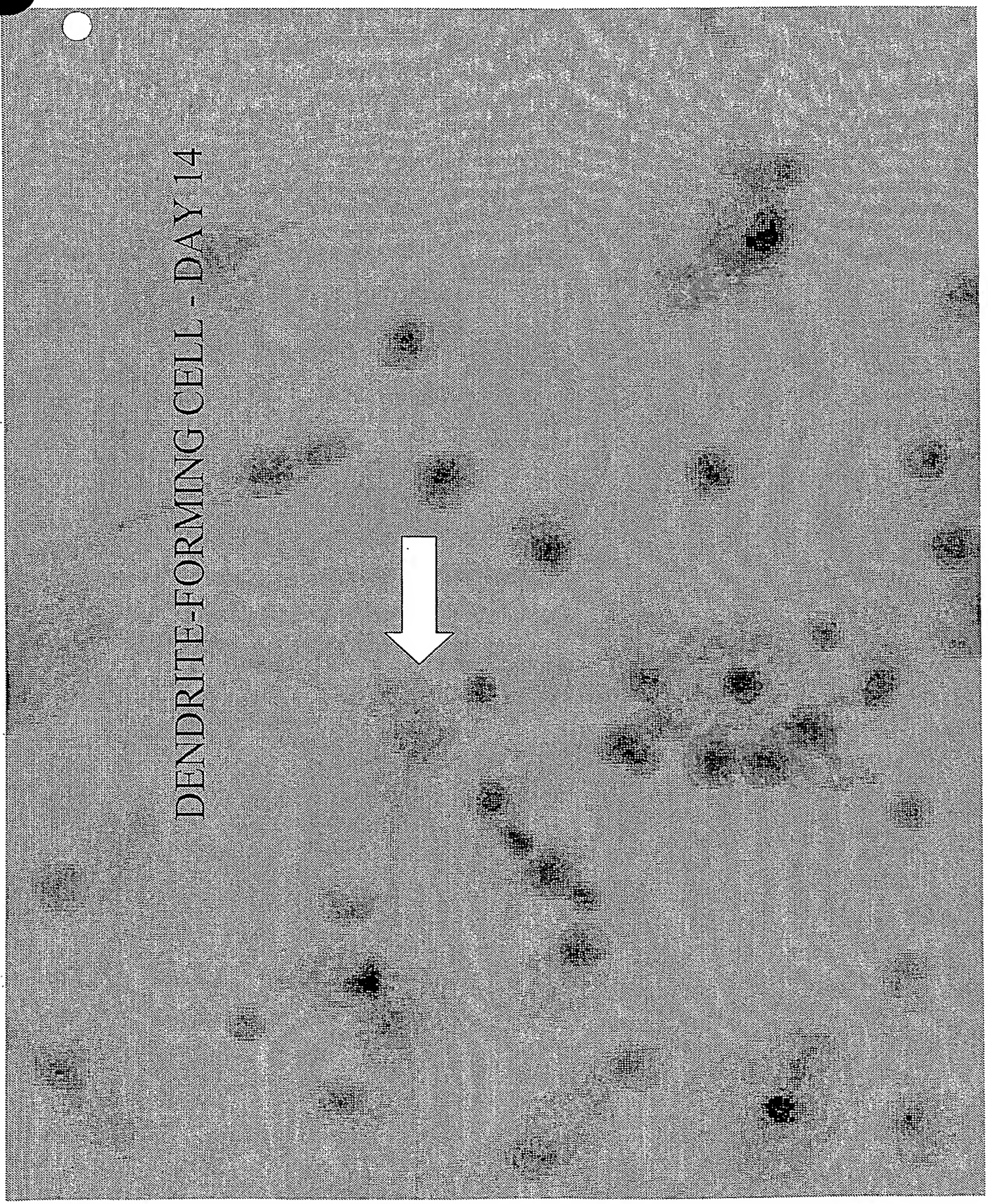

Fig. 10

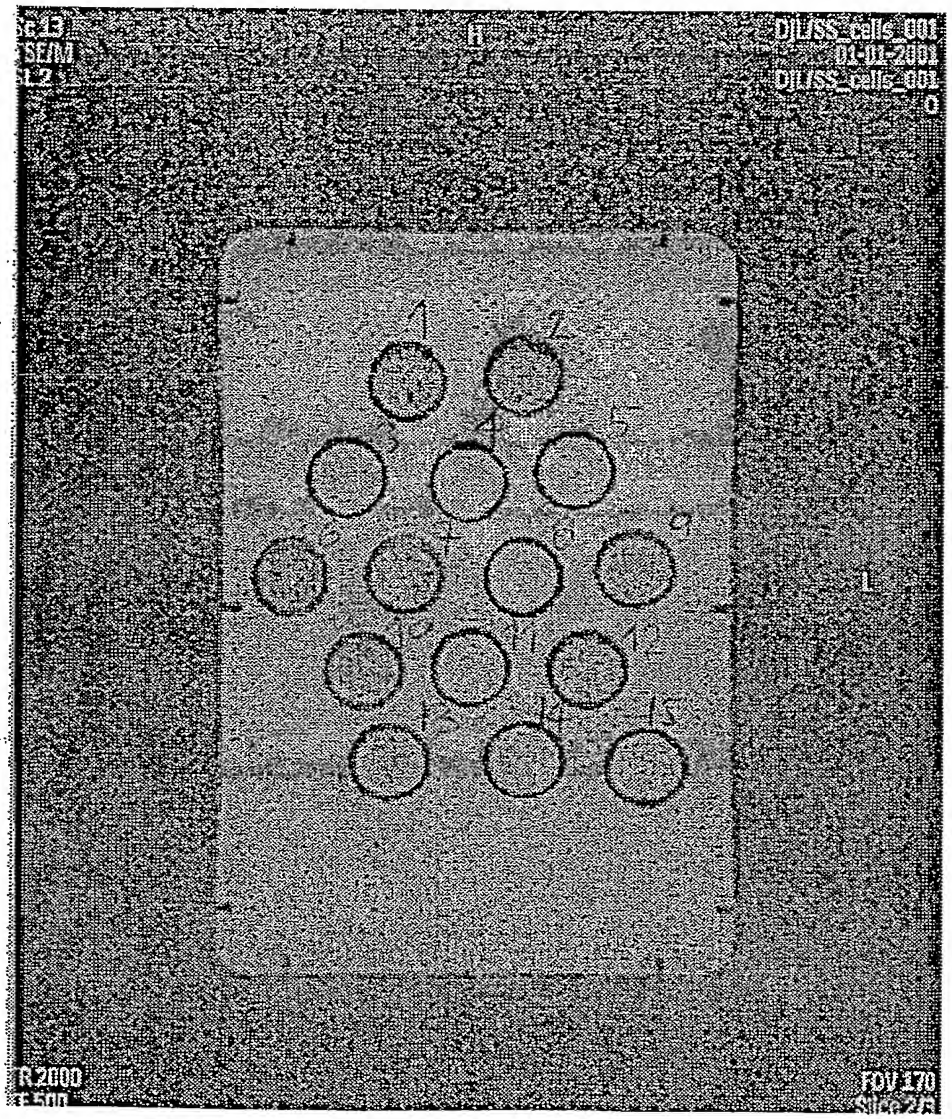

Fig. 11

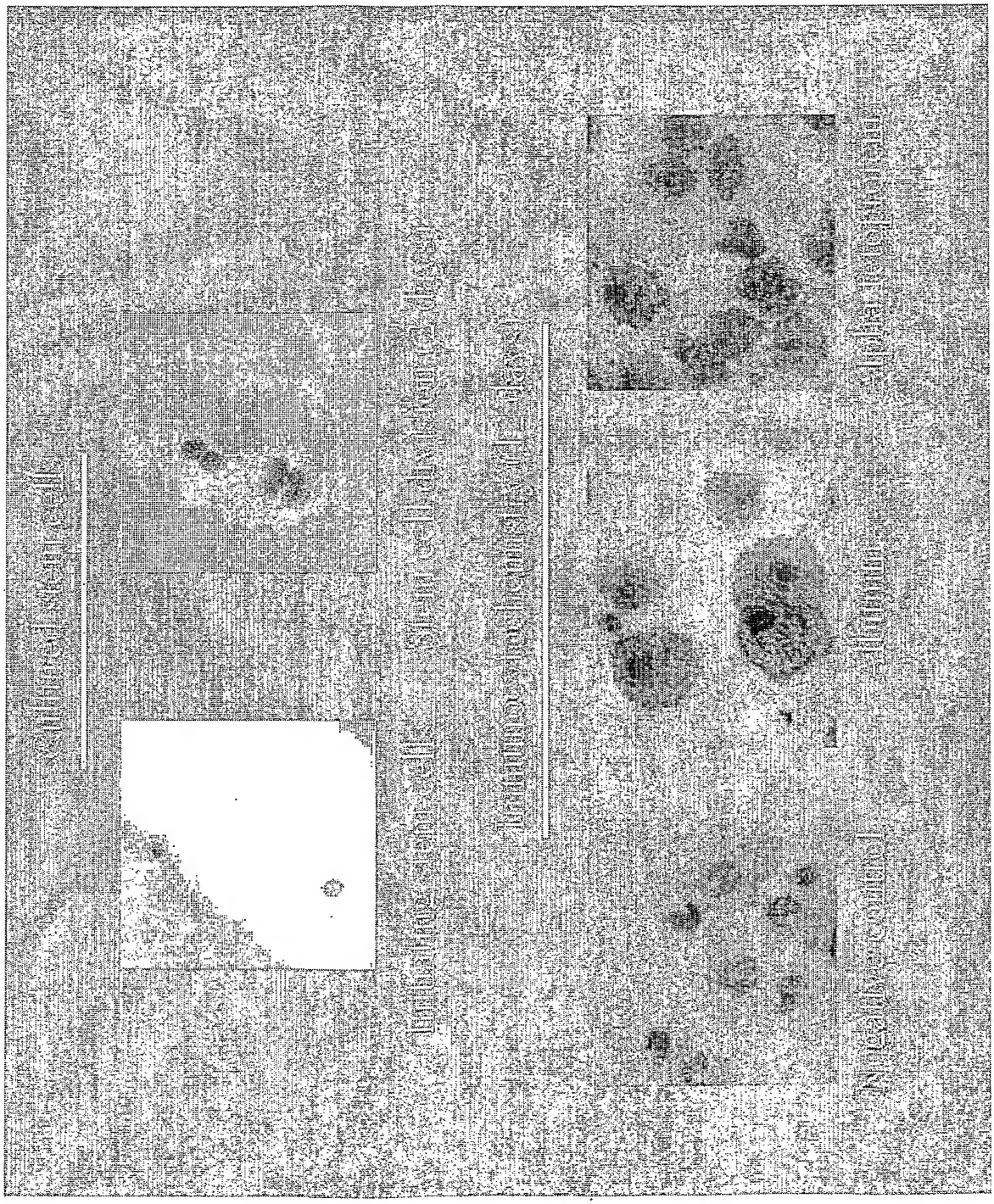

Fig. 12